DNA Methylation and Differentiation of Human Keratinocytes

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DNA methylation is a postreplicative modification thought to play a role in gene transcription in eucaryotes. Differences in the amount of 5-methylcytosine as a function of age and differentiation state have been reported [7, 14–18]. DNA isolated from human skin keratinocytes was analyzed for its 5-methylcytosine content. The 5-methylcytosine in DNA from neonatal and adult human keratinocytes was found to vary as a function of differentiation state. Differentiation of keratinocytes in vitro was promoted using a simple method where keratinocytes were plated directly onto the plastic surface of a culture flask, grown to confluence, and placed on a rocking culture platform that cyclically exposed the cells to air 50% of the time. Terminal differentiation was evident after approximately three weeks in culture. The 5-methylcytosine content of the DNA from differentiated human keratinocytes was 1.4%, whereas that of undifferentiated human keratinocytes was 3.1%. No difference in the 5-methylcytosine content of DNA as a function of the age of the donor was found. *J Invest Dermatol 93:687–690, 1989*

The DNA of most organisms is methylated after synthesis. In all higher eucaryotes, 5-methylcytosine is the only minor base that is found. It makes up 2%–14% of the total cytosine found in a genome—the amount varies widely with the organism [1–3].

Various functions have been proposed for the methylation of DNA [4]. In procaryotes, methylation acts to protect DNA against enzyme restriction [5]. The exact role of methylation in eucaryotic DNA is not established. Evidence exists to support a role for methylation in DNA repair, aging, oncogenesis, and cell differentiation [6–9].

Methylations of eucaryotic DNA are found predominantly in 5′ cytidine phosphoguanosine (CpG) dinucleotide sequences. Approximately 70% of these sequences are methylated in the human genome [1,10,11]. The number of methylated cytosine base sites on DNA has been linked to gene expression. Undermethylated sites are thought to foster gene expression, whereas highly methylated sites result in lack of expression [1,4,12,13].

Age-related changes in the methylcytosine content of DNA are found in bovine thymus and heart where the 5-methylcytosine content of the DNA decreases about 25% by the age of 12 years [7]. Similar changes were reported for rodents where the methylcytosine level was seen to decrease at a rate of 0.012% per month [14].

Variation in overall DNA methylation between differentiated and undifferentiated cells has been reported. Erythroleukemia cells are induced to differentiate and produce hemoglobin in response to hypomethylating agents. The overall methylation of the DNA was found to decrease from 3.7% to 2.1% 5-methylcytosine in Friend leukemia cells when uninduced cells were compared with those induced to synthesize hemoglobin with 5-aza-2′-deoxycytidine [15]. Human cell line K562 can be significantly hypomethylated by treatment with the methylation inhibitor, methinin [16] and concomitantly differentiated to produce hemoglobin. Additionally, confluent BHK21 cells in culture were shown to have a decreased level of DNA methylation when compared with actively growing cells [17]. However, a similar study of confluent and nonconfluent skin fibroblasts showed no difference in the methylcytosine content of the cellular DNA [18].

The epidermis presents a continuously differentiating system as the basal cells proliferate and migrate upward. Morphologically, the keratinocytes flatten in the upper layers and form desmosomal junctions and phagocytic vesicles. The 8-nm intermediate keratin filaments aggregate and form bundles [19]. The differentiated keratinocytes found in the suprabasal layers of the epidermis synthesize high molecular weight keratins (66–68 kd) [20], whereas lower molecular weight keratins (45–58 kd) are expressed by both the basal and suprabasal layers. The changes in keratins found from the basal to granular layers have been correlated to changes in appearance of specific keratin mRNA for these new keratins [21].

Keratinocytes grown in conventional submerged culture systems are hyperproliferative with respect to the synthesis of keratins and other proteins. Only the lower molecular weight keratins are synthesized by these cultures [21,22]. Many attempts have been made to establish an in vitro cell culture system in which differentiated keratinocytes stratify to form a multilayer cell system resembling skin. Complete stratification and differentiation of keratinocyte cultures are seen in a system where keratinocytes are seeded on a collagen/fibroblast raft. After the keratinocyte culture is confluent the raft is raised to the air-media interface [23–25]. The keratinocytes are nourished by diffusion of the media through the collagen. The cells form layers and express high molecular weight keratins (66–68 kd), both indicative of a differentiated state [23].

In this study, a method was developed whereby keratinocytes were differentiated by cyclic exposure of the cultured cells to an air environment without the use of a dermal equivalent. Using these

Manuscript received July 6, 1988; accepted for publication February 2, 1989.

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Abbreviations:
ATP: adenosine triphosphate
CpG: cytidine phosphoguanosine
DNA: deoxyribonucleic acid
EDTA: ethylenediaminetetraacetic acid
kd: kilodaltons
SDS: sodium dodecylsulfate

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cells, the genomic 5-methyldeoxycytidine of human keratinocytes as a function of their differentiation and age was quantitated.

MATERIALS AND METHODS

Materials [γ-^{32}P]ATP (5000 Ci/mmol) was purchased from New England Nuclear, monophosphate nucleotide standards from Pharmacia, Triton X-100 from Sigma, and molecular biology grade phenol from Boehringer Mannheim Biochemicals.

Keratinocyte growth media and some human keratinocyte cultures were purchased from Clonetics Corporation (Denver, CO). Trypsin-EDTA was purchased from Gibco, as was the trypsin and soybean trypsin inhibitor. The human fibronectin was obtained from Meloy Labs.

Deoxyribonuclease-free ribonuclease, micrococcal nuclease, nuclease P1, phosphodiesterase, and proteinase K were purchased from Sigma. T_4 polynucleotide kinase was obtained from International Biotechnologies Inc.

Cell Culture Human keratinocytes were cultured from neonatal foreskin and adult surgical skin, or adult breast cells in second passage from primary culture, supplied by Clonetics (Denver, CO).

Epidermal cell suspensions were prepared as described by Gilchrest [26]. The human epidermal cells were resuspended in keratinocyte growth medium (Clonetics), which is a modified MCDB 153 formulation supplemented with epidermal growth factor(20/ng/ml), bovine pituitary extract(10/μg/ml), insulin (1 μg/ml), hydrocortisone(10 μg/ml), and 0.03 mM calcium. A 25-cm^2 flask coated with a layer of human fibronectin (0.1 mg/ml) was seeded with 10^5 cells and maintained in an incubator at 5% CO_2 and 37°C, and the medium was replaced every 2–3 d. Undifferentiated basal cells were grown by maintaining the cells at 50%–80% confluency in the keratinocyte growth medium through serial passaging.

Differentiation of Keratinocytes In Vitro The keratinocytes were plated on fibronectin-coated 75-cm^2 culture flasks and when confluent, were fed keratinocyte growth media supplemented with 2 mM calcium. The flasks were then put into a controlled atmosphere chamber (Bellco Glass Company, Vineland, NJ), and the chamber was put on a rocker platform. The chamber rocked at eight cycles per minute exposing the cells in the flask to the air half of the time and bathing them in the media the other half. The cells were grown in air at 37°C; the atmosphere was changed every day and the media was changed every two or three days.

Electron Microscopy The keratinocyte cultures were fixed in situ with 3% phosphate buffered glutaraldehyde, postfixed with 1% osmium tetroxide, and embedded in Epon 812 resin. Cross sections were stained with uranyl acetate and lead citrate and photographed in a JEOL 100B electron microscope [27].

Isolation of DNA Keratinocytes were lysed and proteins digested by treatment for 3 h at 37°C with a solution of 0.5M EDTA, 100 μg/ml proteinase K, 1% Triton X-100.

The DNA was extracted with phenol/chloroform, treated with deoxyribonuclease-free ribonuclease (0.1 mg/ml) for 3 h at 37°C, and reextracted with phenol/chloroform. The DNA was collected by centrifugation after ethanol precipitation [28].

The DNA was quantitated spectrophotometrically by absorbance ratio at 260/280 nm. The DNA samples were stored at 20°C until analyzed for cytosine base methylation.

DNA Methylation Quantification The DNA base methylation was quantified using a ^{32}P-postlabeling technique [29]. This involves digestion of DNA into 3′-monophosphate deoxynucleotide fragments by incubating approximately 5 μg of a DNA sample with micrococcal nuclease (5 units) and phosphodiesterase (0.3 units) for 2–3 h at 37°C. Both ends of the fragments were then radiolabeled by incubation of 1 μg of the DNA sample with 20 μCi [γ-^{32}P]ATP (5000 Ci/mmol) and T_4 polynucleotide kinase (5 units) in a pH 9.5 solution of 10 mM bicine-NaOH, containing 50 μM ATP, 10 mM magnesium chloride, 1.0 mM spermidine, and 10 mM dithiothreithol for 3 h at 37°C. The resulting 3′,5′-biphosphate deoxynucleotides were then incubated with nuclease P1 for 1 h. In this step, the 3′-[^{32}P] end of the fragments were cleaved leaving 5′-[^{32}P]-monophosphate deoxynucleotides.

The 5′-monophosphate deoxynucleotides were separated isocratically by high pressure liquid chromatography in 0.1 M sodium phosphate buffer at 1.0 ml/min on an octadecyl (^{18}C) IBM column at 37°C. The radioactive samples were spiked with cold 5′-monophosphate deoxynucleotides, and the ultraviolet detector was set at 254 nm to detect the cold markers. Fractions were collected coinciding with the ultraviolet absorbance peaks of the cold nucleotides and quantitated by liquid scintillation.

Keratin Extraction and Electrophoresis To prepare samples for electrophoresis, the cells were harvested by centrifugation at 1000 × *g* for 5 minutes after treatment with 0.25% trypsin at 37°C for 5–10 min. The cells were suspended in 50 mM Tris-Cl buffer, pH 7.8, 1% Triton X-100, homogenized with a Dounce homogenizer, and centrifuged at 10,000 × *g* for 10 min. The resulting pellet containing the keratins was solubilized by vortexing and boiling in 10 mM Tris pH 8.5, 1% SDS, 0.2 M mercaptoethanol buffer for 5 min. The cellular debris was separated from the solubilized keratins by centrifugation at 10,000 × *g* for 10 min.

The epidermis of human skin, obtained from plastic surgery, was removed by peeling after submersion in 60°C water for 2 min [30]. The epidermis was homogenized and the keratins extracted as described.

SDS gel electrophoresis was performed on 7.5% polyacrylamide gels. The gels were stained with Coomassie blue R-250 and destained with a 10% acetic acid/50% methanol solution. Standards used for determination of molecular weight were: phosphorylase B (97 kd), bovine serum albumin (66 kd), ovalbumin (43 kd), and carbonic anhydrase (31 kd).

RESULTS

Morphology of Cultured Keratinocytes Human keratinocytes proliferated to create and maintain a confluent monolayer when cultured in keratinocyte growth media. When keratinocyte growth media was supplemented with calcium to a concentration of 2 mM, the confluent monolayers of cells, maintained in stationary culture, formed discreet piling colonies, but produced no more than two cell layers.

When confluent cultures with media containing 2 mM calcium were exposed to air 50% of the time by placing the flasks on a rocking platform, the keratinocyte cultures differentiated during 2–3 wk of growth. Multiple layers of cells encompassing more than 80% of the total area of the flask were visible. Electron microscopy revealed that multiple layers of cells were present. A number of characteristics of a differentiated keratinocyte system were seen (Fig 1). The upper layers appeared flattened when compared with the basal layers. Desmosomes were apparent between the cells and keratin filament bundles were present. The cornified cell envelope and densely packed keratin bundles can be seen in the sloughing cells of the cultures (Fig 1*B*).

Keratin Expression in Cultured Keratinocytes Keratins synthesized in undifferentiated and differentiated rocker cultures of adult human keratinocytes were analyzed by one-dimensional SDS gel electrophoresis. The differentiation specific keratin K1 (66–68 kd) of the epidermis was synthesized in the keratinocytes differentiated in the rocking cultures (lane *b*, Fig 2). Keratin samples from confluent keratinocyte monolayers grown in a stationary culture in media containing 2 mM calcium contain the lower molecular weight keratins but do not contain K1, the 66–68-kd keratin(lane *c*, Fig 2). In addition, the keratin K1 was not expressed in stationary confluent cultures of keratinocytes grown at 0.03 mM calcium.

DNA Methylation as a Function of Differentiation The 5-methyldeoxycytidine content of DNA was determined in DNA isolated from human keratinocytes. The 5-methyldeoxycytidine content of the DNA, expressed as a percentage of the total cytosine in the DNA, varies as a function of the differentiation state of the cells (Fig 3).

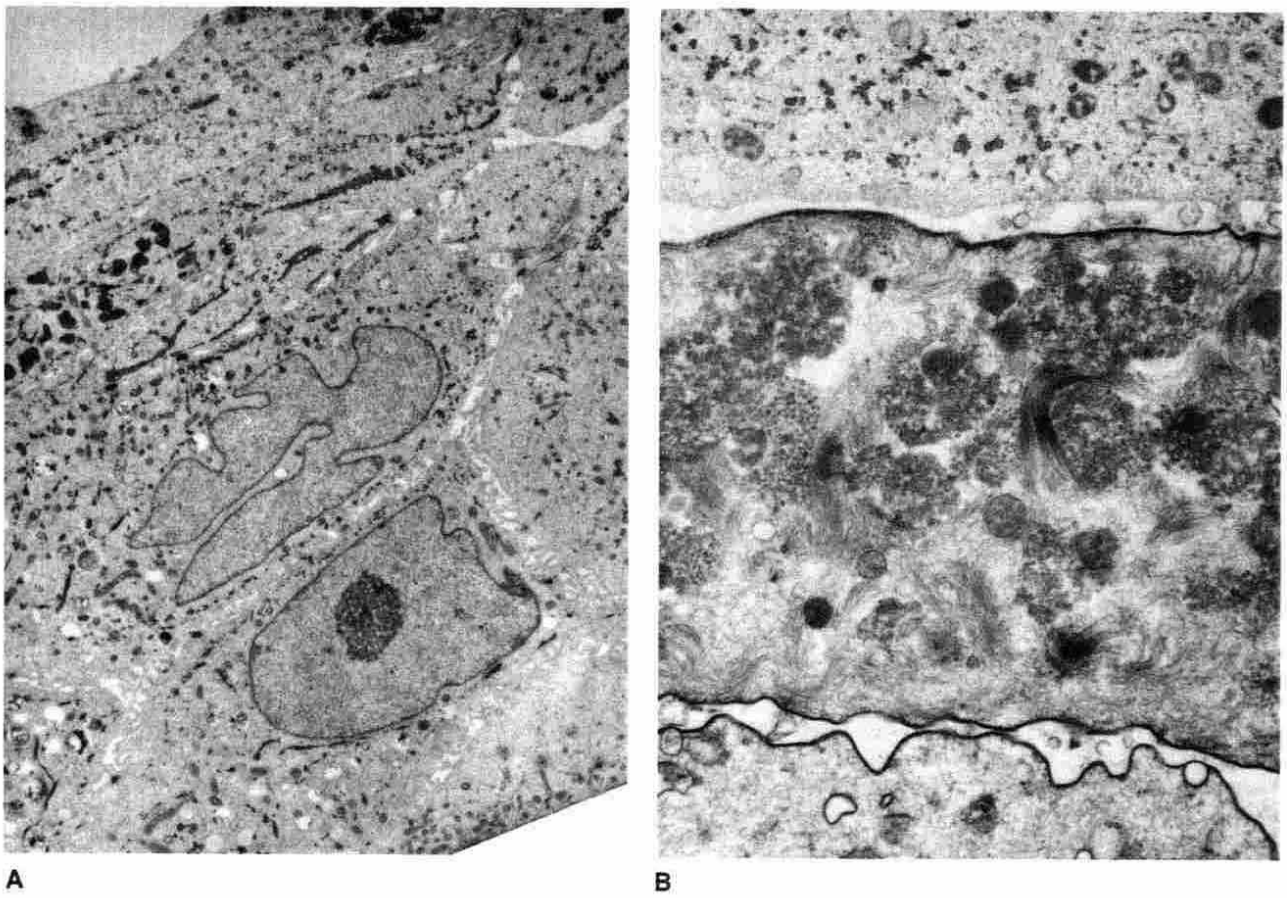

Figure 1. Morphology of human epidermal cells grown in rocking culture. *A*) Multiple cell layers are evident with the upper cell layers flattened. The intermediate cell layers contain keratin filament bundles (F) and desmosomes (D). *B*) Sloughing corneocytes contain bundles of keratin filaments and are surrounded by a corneocyte envelope. (*A*, ×6666; *B*, ×16666).

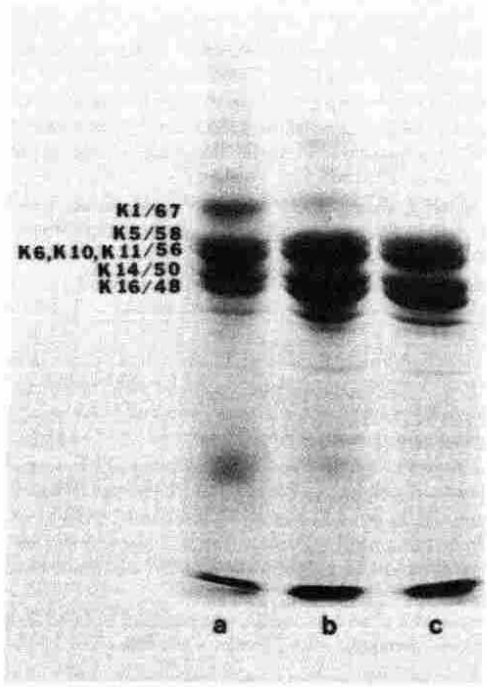

Figure 2. Keratin expression in human epidermal cells grown in rocking culture. *a*) Keratinized epidermis, *b*) keratinocytes grown in rocking culture, *c*) keratinocytes grown in submerged confluent culture in media containing 2 mM calcium. The Moll-Franke number and molecular weight of the major keratins are shown at the left of the gel in kilodaltons.

The average level of 5-methyldeoxycytidine in the undifferentiated adult was 3.1% compared with differentiated adult keratinocyte levels, which averaged 1.0% 5-methyldeoxycytidine. This same difference between undifferentiated and differentiated was also found in the neonatal cells which averaged 3.1% 5-methyldeoxycytidine for the undifferentiated and 1.1% for the differentiated population. These differences in percentage of methylation in both groups are statistically significant, using a *t*-distribution for independent small samples ($p < 0.005$).

Keratinocytes which reached confluence but were not exposed to air had a 5-methylcytosine content representative of undifferentiated cells. The mean 5-methylcytosine content of these cells was 3.4%.

DNA Methylation as a Function of Age There is little difference in the methylation levels of the adult and neonatal DNA samples. The mean values for the DNA from adult and neonatal keratinocytes are very similar and the differences are not statistically significant (Fig 3). The mean 5-methyldeoxycytidine content of the adult differentiated DNA is 0.99% compared with a mean of 1.1% for neonatal differentiated DNA. The means for methyl cytosine from undifferentiated keratinocytes are 3.14% for the adult-derived cells and 3.17% for the neonatal cells.

DISCUSSION

The DNA of differentiated keratinocytes contains less 5-methylcytosine than the DNA of undifferentiated keratinocytes. The decrease from 3.0% to 1.0% 5-methylcytidine is a significant change in the methylation state of the DNA with differentiation. Concomitant with hypomethylation and differentiation, the keratin (K1) having a molecular weight of 66–68 kd was synthesized in the terminally differentiated keratinocytes.

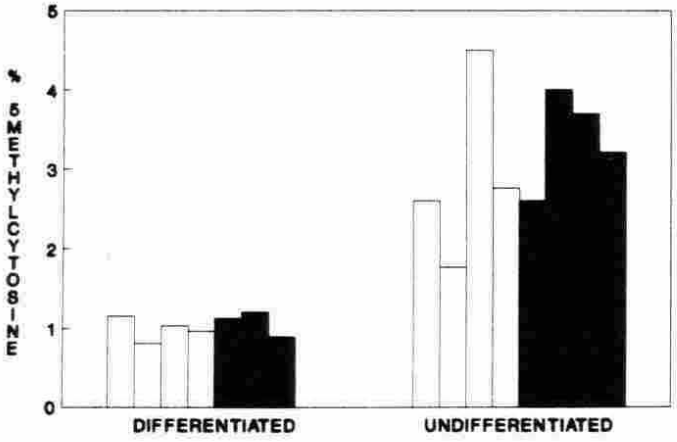

Figure 3. The 5-methylcytosine content of undifferentiated and differentiated human keratinocytes. The 5-methylcytosine content of the DNA is expressed as a percentage of total cytosine. The *open bars* represent adult keratinocyte cultures, while the *cross-hatched bars* represent neonatal cultures.

Similar changes in DNA methylation have been observed in erythroleukemia cells that are induced to produce hemoglobin by hypomethylating agents. Treatment of Friend erythroleukemia cells with 5-aza-2′-deoxycytidine results in a decrease in overall methylation of cytosine from 3.67% to 2.14%. The treatment results in the synthesis of hemoglobin, which is the differentiated gene product of these cells [15]. Treatment of human erythroleukemia K562 cells with another hypomethylating agent, methinin, resulted in a decrease from 5.1% to 2% 5-methylcytosine. These cells produced hemoglobin in response to this treatment [16].

No significant change in the 5-methylcytosine content of the DNA with age was observed. Keratinocytes derived from adult skin and neonatal skin showed no difference in the 5-methylcytosine content of the DNA. Previous reports have shown decreases in the 5-methylcytosine content of rodent and bovine organs with age [7,14].

In this study, a novel method for promoting the differentiation of keratinocytes in vitro was introduced. Previous literature indicated a need for both a dermal equivalent system on which the keratinocytes are plated, and an emergence of the cells to the air-media interface. The method used in this study eliminates the need for a dermal equivalent. The cells are grown directly on the plastic surface of a culture flask. Exposure of the cultured cells to the air 50% of the time, along with the supplemented calcium, appears to be sufficient to initiate the differentiation of the keratinocytes. Higher molecular weight keratins are synthesized and the cells stratify into many layers.

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